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Expression Profile of Acetyl CoA Carboxylase Beta (*ACACB*) Gene during the Pre and Post-hatch Period in Chicken

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ABSTRACT

Background: Acetyl-CoA Carboxylase Beta (*ACACB*) plays a key role in fatty acid oxidation and was known to be involved in production of very-long-chain fatty acid and other compounds needed for proper development. This gene is mainly expressed in the tissues of heart, muscle, liver and colon. It chiefly involved in the production of malonyl-coA, a potent inhibitor of carnitine palmitoyl transferase I (CPT-I) enzyme needed in transport of long-chain fatty acyl-coAs to the mitochondria for β-oxidation.

Methods: The present study was conducted to explore the expression pattern of the *ACACB* gene in breast muscle tissue during pre-hatch embryonic day (ED) 5th to 18th and post-hatch (18th, 22nd and 40th week of age) periods of White leghorn (IWI line) by using Quantitative real-time PCR (qPCR). Then, fold change of *ACACB* gene expression was calculated.

Result: Our study showed that the *ACACB* gene expression was down-regulated during embryonic stages from ED6 to ED18. The gene expression was also down-regulated during adult stages *i.e.* on 22nd and 40th week of age. This result indicated that the initial expression of the *ACACB* gene is required for embryo development and during adult periods, low gene expression leads to the less fat deposition in muscle of layer chicken. Finally, it can be concluded that there was a differential expression pattern of the *ACACB* gene during the pre-hatch embryonic and post-hatch adult periods to mitigate varied requirements of lipids during different physiological stages in layer chicken.

Key words: *ACACB* gene, IWI chicken, Pre and post-hatch, qPCR.

INTRODUCTION

For energy metabolism, fatty acids are essential constituents and important substrate for all biological membrane lipids. They contribute to the regulation of a wide variety of biological activities. Long-chain fatty acid were broken down in to smaller acetyl coA by fatty acid oxidation or mitochondrial beta (β)-oxidation pathway. The Acetyl-CoA Carboxylase Beta (*ACACB* or *ACC-β* or *ACC2*) plays a key role in β-oxidation pathway. It was located on chromosome 15 of chicken (NCBI). *ACACB* attaches to the outer-leaf of mitochondria through a stretch of hydrophobic sequence (Abu-Elheiga *et al*., 1997; Abu-Elheiga *et al*., 2000; Abu-Elheiga *et al*., 2005). Acetyl-CoA carboxylase (*ACC*) is the rate-limiting and biotin-containing enzyme catalyzing the ATP-dependent carboxylation of acetyl-CoA to produce malonyl-CoA (Abu Elheiga *et al*., 2001; Tong and Harwood 2006). Two major isoforms of *ACC*, *ACACA* (Acetyl-CoA carboxylase A) and *ACACB*, which is an intermediate substrate that plays a pivotal role in the regulation of fatty acid metabolism and it is the rate-limiting step in fatty acid synthesis. Both *ACC* genes are under the control of multiple promoters, which are regulated by diet and hormones. Especially, a fat-free carbohydrate rich diet induces the synthesis of *ACACA* and *ACACB* and increases their activities. Starvation represses the expression of the carboxylase genes and decreases the activities of the *ACC* enzymes (Kim 1997). Rosebrough *et al*. (2008) and Rosebrough *et al*. (2011) found that feeding broilers a diet containing a high crude protein (CP) level suppressed the mRNA expression of acetyl coenzyme carboxylase (*ACC*), in a comparison of low-protein and high-protein diets. Santoso *et al*. (1993) suggested that quantitative feed restriction reduces fat deposition by inhibiting the activity of the rate-limiting enzyme during lipogenesis (*ACC*) in the livers of broiler chickens, similarly Tan and Othani (2000) found in White Pekin ducks. Ji *et al*. (2012) studied avian adipose tissue transcriptome and metabolism in chicken under two different diets, one under fasting and the second under insulin deprivation in commercial chicken and observed that several genes were up regulated in fasting,

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including the fatty acid oxidation gene *ACACB* suggesting its role in fat metabolism. *ACACB* gene was mostly expressed in non-lipogenic tissues such as skeletal and heart muscle and to a lesser extent in the liver (Abu-Elheiga *et al*. 1995; Lopaschuk *et al*. 1994; Ha *et al*. 1996; Abu-Elheiga *et al*. 1997). The *ACACB* generated malonyl-CoA that functions as an inhibitor of the carnitine/palmitoyl-transferase 1 (CPT1) activity and the transfer of the fatty acyl group through the carnitine/palmitoyl shuttle system to inside the mitochondria for â-oxidation (McGarry *et al*. 1978; Ruderman *et al*. 1999; Wakil and Abu-Elheiga 2009). The net result is reduced fatty acid oxidation and increased fatty acid and triglyceride synthesis, at the expense of glucose utilization.

High fat content in the meat exert adverse effect on human health (Milićević *et al.* 2014) and its accumulation in excess amount exert a negative impact on reproductive performance of birds (Xing *et al.* 2009). The expression level of this particular gene at different age groups will form the base for understanding its action and subsequently used for manipulating the gene functionality to reduce fat deposition. Therefore, the present study was conducted to know the expression levels of the *ACACB* gene during the pre and post-hatch period in the white leghorn IWI line.

MATERIALS AND METHODS

Experimental birds

The present study was conducted in IWI chicken line of the White Leghorn breed which is maintained at ICAR-Directorate of Poultry Research, Hyderabad, India during the year 2020. The birds were provided *ad lib* feeding, watering and standard managemental practices for obtaining good performance with proper welfare (Chatterjee *et al.* 2008; Rajkumar *et al.,* 2010). This line was undergoing index selection for higher egg production up to 64 weeks of age.

Sample collection

Whole embryos from embryonic days (ED) ED5 to ED8 (n=4 per day) and breast muscle tissues from ED9 to ED18 (n=4 per day) were collected for the study. Also, before (18^{th} week) and after (22^{nd} and 40^{th} week) onset of egg production, 4 birds each were sacrificed and the breast muscle tissues were collected under aseptic conditions following the approved protocol of Institute Animal Ethics Committee (IAEC) of ICAR-Directorate of Poultry Research, Hyderabad. All the samples were collected in 1.5 ml Dnase-RNase-free sterile polypropylene tubes using DEPC (0.1%) treated sterile instruments. They were chilled immediately on ice in order to minimize RNA degradation, transferred to the lab and kept at -80°C until further use.

RNA extraction

Total RNA was isolated from embryo and muscle tissues using Trizol (Amresco), according to the standard protocol (Badola *et al*., 2004; Mishra *et al.,* 2008; Pal *et al*., 2011). Resulted RNA pellet was re-suspended in 50µl nuclease-free water and the concentration and quality were determined in Genova plus NanoDrop spectrophotometer and 1.2% formaldehyde agarose gel. The RNA sample showing the OD_{260}:OD_{280} values in between 1.8 to 2.2 were considered as good quality and were used further.

First strand cDNA synthesis

Each sample of RNA was treated with DNaseI (Fermentas) and converted in to cDNA using Verso cDNA synthesis kit (Thermo Scientific, #00775881) following standard protocol (Bhattacharya *et al.,* 2011a). This reverse transcription was carried out in thermocycler (Himedia) using the components 5X cDNA Synthesis Buffer (4 µl), Anchored Oligo dT (0.25 µl), Random Hexamer (0.75 µl), dNTP Mix (2 µl), RT Enhancer (1 µl), Verso Enzyme Mix (1 µl), RNA template (RNA) (2 µg) and Nuclease-free H_2O (make up the volume to 20 µl) under the condition 42°C @ 30 minutes and 95°C @ 2 minutes. The resulted cDNA was stored at -20°C until further use. The *ACACB* and *GAPDH* genes were amplified by using cDNA as a template in RT-PCR with gene specific primers and were then confirmed by 1% agarose gel electrophoresis (Fig 1). The *GAPDH* gene was used as internal control for quantification of *ACACB* expression in realtime PCR (Bhattacharya *et al.,* 2011b).

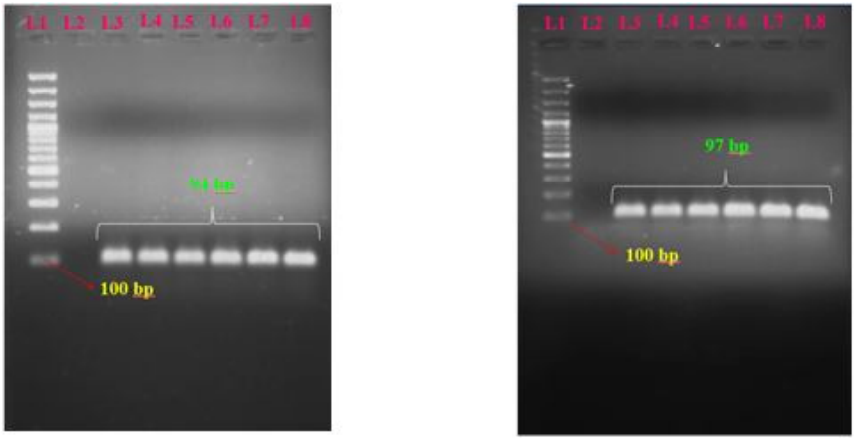

Fig 1: Agarose gel electrophoresis of qPCR amplified product of *ACACB* (94 bp) (a) and *GAPDH* (97 bp) (b) genes; Lane1: 100 bp ladder Plus; Lane 2: Negative control; Lanes L3-L8 in both (a) and (b): Amplified qPCR products.

Real-time quantitative PCR (qPCR)

Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) was used as an internal control for normalizing different amounts of input RNA. Intron-spanning primers used for qPCR expression were designed using the IDT Primer Quest software (Table 1). The mRNA expression levels of the target (*ACACB*) and reference (*GAPDH*) genes were quantified by using thermal cycler Applied Biosystems® Step One Real-time PCR (Life Technologies) with BrightGreen 2x qPCR MasterMix-ROX (abm). All the PCR reactions were performed in three biological replicates with a final volume of 10µl containing 5µl of BrightGreen 2x qPCR MasterMix-ROX, 0.5µl of each forward and reverse primer (10 µM), 1µl of cDNA and 3µl of nuclease-free water. Thermal cycling conditions followed were initial denaturation at 95^0C for 10 minutes followed by 40 cycles of PCR stage (95°C for 15 seconds and 58°C for 1 minute) and melting stage (95°C for 15 seconds, 60°C for 1 minute and 95°C for 15 seconds) was performed at the end of the qPCR to check the specificity of amplification.

Relative quantification

Comparative Ct method was used for estimating the expression levels of gene (Livak and Schmittgen 2001; Bhattacharya and chatterjee 2013). Threshold cycle (Ct) values of *ACACB* and *GAPDH* genes were obtained after performing qPCR. These Ct values were used for estimating the "n-fold up/down-regulation of transcription" and *ACACB* gene expression levels relative to the internal control by using the following formula;

$$\text{Fold of expression} = 2^{-\Delta\Delta Ct}$$

Where,

ΔCt = Average Ct of the target gene (*ACACB*) - Average Ct of the reference gene (*GAPDH*)

$\Delta\Delta$Ct = Average ΔCt of the target sample – Average ÄCt of the calibrator sample.

RESULTS AND DISCUSSION

Acetyl-CoA carboxylase (*ACC*), a rate-limiting enzyme was known to involve in *de novo* fatty acid synthesis specifically in catalysing process of acetyl-CoA to malonyl-CoA (Wakil 1958; Nugteren 1965; Abu-Elheiga *et al*. 2001). Two forms of *ACC* genes were described such as *ACACA* located in the cytosol and *ACACB* associated/linked/connected with the mitochondrial membrane (Munday 2002). The *ACACB* gene was known to be associated with nephropathy, obesity, diabetes and end-stage renal diseases (Tang *et al*. 2010; Riancho *et al*., 2011; Zain *et al*. 2017). This gene was first discovered in rat heart (Thampy 1989). Mice lacking *ACACB* gene are reported to be protected against obesity and diabetes (Abu-Elheiga *et al*. 2003). The "Gallus Expression in Situ Hybridization Analysis" (GEISHA) database revealed non-availability of expression data for the gene. The human protein atlas (https://www.proteinatlas.org/ENSG00000076555-*ACACB*/tissue) showed that the gene expresses highly in skeletal muscle tissue compare to all other tissues. Hence, in chicken, skeletal muscle is targeted for its expression analysis.

Thus, we have chosen IWI line i.e. developed from white leghorn breed that was known for high egg production. These egg laying chickens provides good insight to understand various processes of lipid mobilization, transfer and utilization for yolk precursor synthesis, which is important in deposition of yolk and embryo development (Saarela *et al*. 2008). Merkin *et al.* (2012) while studying evolutionary dynamics of gene regulation in mammalian and bird tissues, indicated that *ACACB* gene expressed at low to medium level in colon, heart, kidney, lung, skeletal muscle tissue, spleen and testis while its expression is below cut off level in brain and liver. Similarly, a study by Barborasa-Morais *et al.* (2012) also showed that *ACACB* gene is low to medium in kidney, skeletal muscle and heart and below cut-off in brain and liver. There was no clear-cut idea how the gene is expressed over different developmental stages of chicken. There was no reports are available on expression of the *ACACB* gene during the pre and post-hatch period in chicken. However, some other genes are studied and reported during pre and post-hatch period in chicken. In domestic chicken, the higher embryonic growth was reported during late embryonic stage (Cogburn *et al*. 1989). In both control broiler and Aseel, the *ACTRIIB* gene expressed at ED7, ED11 and ED16 (Vishnu *et al*. 2017). Similar results were also found in the post-hatch period in layer chicken line, where the highest and lowest expression level of the *SCD* gene was found on the 2nd (40.75) and 6th week (27.75), respectively (Sagar *et al*., 2019). In the control layer, the expression of *ACACA* gene was up-regulated during 2nd, 4th and 6th weeks of age, when compared to the day one post-hatch period (Prasad *et al*. 2018b). In two indigenous breeds *i.e.* Ghagus and Aseel, the myoglobin (*Mb*) gene expression was studied in different organs like muscle, bursa, heart, spleen and gizzard at day old and 4th week, respectively (Prasad *et al*. 2019). In both Aseel and control broiler, the *ACTRIIB* gene expression was studied and showed up-regulatedon day one and downward upto 4th week and gradually increased at 6th week, respectively (Vishnu *et al*. 2017). In Ghagus, the expression of *TNNC1*

Table 1: Primer details for amplification of genes.

Gene	Primer Sequences (5'-3')	Amplicon Size (bp)	Annealing temp (°C)
ACACB	F: GCTCCTGCTGCCCATATATTA	94	58
	R: GTCCGTGATGACACCTTTCT		
GAPDH	F:ATGGGAAGCTTACTGGAATGG	97	58
	R:TCATCATACTTGGCTGGTTTCT		

gene was studied in different organs such as muscle, bursa, heart, spleen and gizzard, respectively (Prasad *et al*. 2018a). In this context, we explore the expression profile of *ACACB* gene during pre and post-hatch period of IWI line as it no far reported.

Standard curve was plotted to optimize real time PCR, where a regression coefficient (slope) of -2.941 and -4.661 and coefficient of determination (R^2) of 0.9543 and 0.8374, respectively, was found for *ACACB* and *GAPDH* genes (Fig 2). A significant regression coefficient was found in the target and reference genes representing an optimum standardization of gene expression before carrying out the actual experiments. The amplification plot and dissociation curve of the respective target and reference genes were analyzed by qPCR (Fig 3; Fig 4). The common comparative threshold method of analysis *i.e.* $2^{-\Delta\Delta Ct}$ was used to study the relative gene expression during pre and post-hatch period of IWI line. The ED5 and 18th week stage were used as a calibrator to determine the relative expression during pre and post-hatch periods, respectively. A significant ($P<0.05$) effect of *ACACB* gene expression was observed in IWI line at different stages during different developmental periods (Fig 5; Fig 6). The expression of the *ACACB* gene was down-regulated during embryonic period i.e. ED6 to ED18 (on ED6 by 0.40, ED7 by 0.57, ED8 by 0.58, ED9 by 0.38, ED10 by 0.57, ED11 by 0.35, ED12 by 0.26, ED13 by 0.24, ED14 by 0.35, ED15 by 0.62, ED16 by 0.93, ED17 by 0.80 and ED18 by 0.83 folds in this chicken line. During adult stage, the expression was also down-regulated by 0.32 fold on the 22nd week and 0.40 fold on 40th week of age. From this result, we may suggest that due to lower expression of the ACACB gene, the abdominal fat deposition is low in layer chicken. Earlier literature also revealed the same trend in mice where mice lacking *ACACB* gene showed decreased fat storage in abdomen (Abu-Elheiga *et al*. 2001). When metabolic fuel in the body is low and sufficient ATP is needed, ACACB pathway is turned off by phosphorylation. Consequently, the level of malonyl-CoA is reduced, which leads to generation of ATP through increased fatty acid oxidation and the decreased consumption of ATP for fatty acid synthesis. Thus, *ACACB* gene expression is important only during *de novo* fatty acid synthesis when lipids and triglycerides are synthesized in the body.

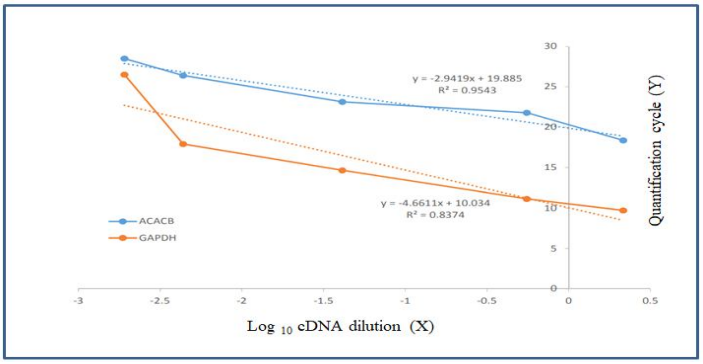

Fig 2: Standard curves of *ACACB* and *GAPDH* genes for standardizing quantitative real time PCR.

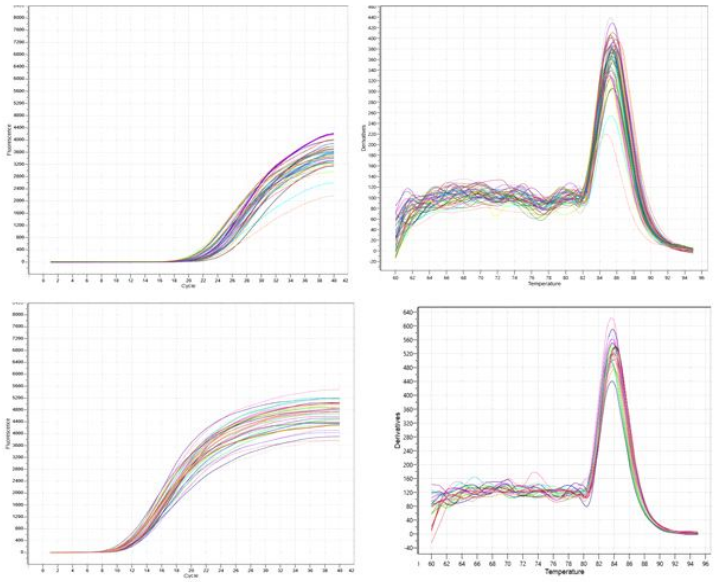

Fig 3: Amplification plot and dissociation curve of *ACACB* and *GAPDH* genes during pre-hatch embryonic period.

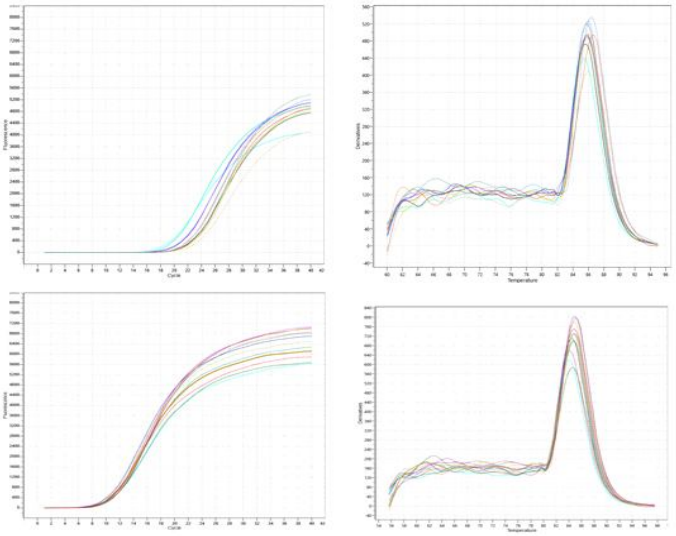

Fig 4: Amplification plot and dissociation curve of *ACACB* and *GAPDH* genes during post-hatch adult stage.

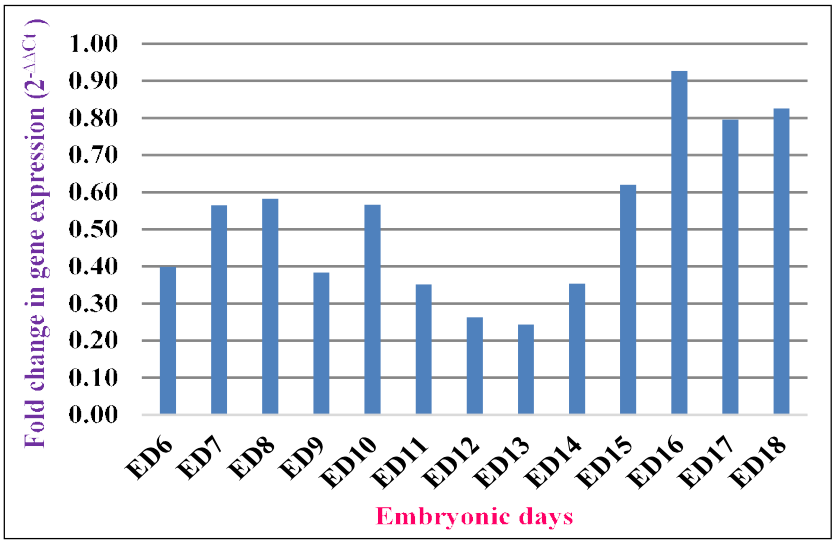

Fig 5: Fold change in expression of *ACACB* gene during pre-hatch embryonic period.

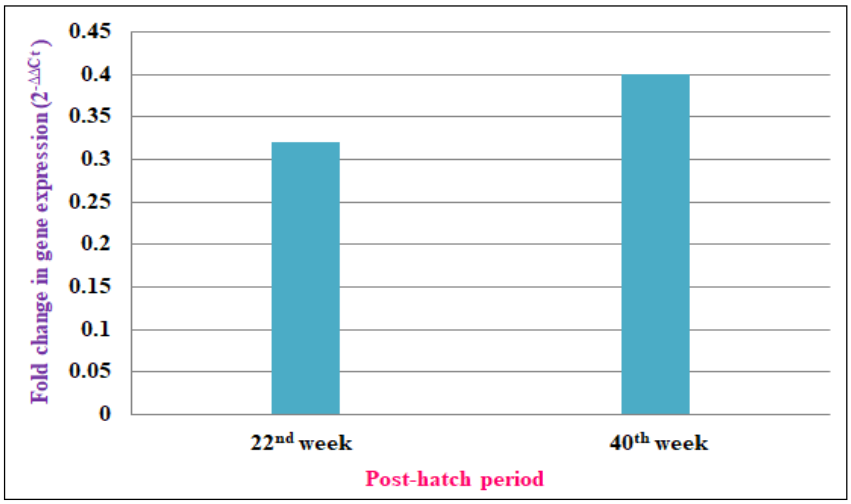

Fig 6: Fold change in expression of *ACACB* gene during post-hatch period.

CONCLUSION

From the results obtained in this study, it can be concluded that there was a differential expression pattern of the *ACACB* gene during the pre-hatch embryonic and post-hatch adult periods to mitigate varied requirements of lipids during different physiological stages in layer chicken.

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